

Appl. No. 10,628,110

Admt. dates Jul. 28, 2003

Reply to Office action of June 9, 2005

ATTACHMENT

Specification with markings:

Method for Rapid Detection of Microorganisms by Changing the Shape of Micro-Colonies

BACKGROUND OF THE INVENTION

[001] *Field of the Invention*

~~The growth of~~ Growing microorganisms in order to detect, enumerate and identify ~~livable~~ viable cells – bacteria, fungi, actinomycetes – is one of the most widely used ~~methods~~ procedures in microbiology. The ability to form a colony on or in an appropriate nutrient media is recognized as the most reliable criteria for cell viability. This requires different biochemical reactions and processes like respiration, transportation, synthesis and decomposing of different proteins, carbohydrates, lipids, enzymes, nucleic acids and many other substances, and creation of inner structures in order to finally reach cell reproduction and creation of a colony.

[002] The detection of live cells cannot be fully substituted by the simple detection of enzymes (enzymatic methods of detection), purified DNA or RNA (PCR methods), antigen – antibody interactions (Enzyme Immunological Analysis, ELISA, Immunoprecipitation, Immunofluorescence, Phagodiagnosics and others), fatty acids

analysis (Chromatography), FTIR spectroscopy or other methods. This is due to the presence of specific molecules or even some working systems of the cell, which can be found in dead cells, cells under deadly stress, or cells without access to some required substances that are therefore restricted in growth potential.

[003] The growth occurs on either solid or liquid artificial or natural nutrient media. The hundreds of different media for total count and ~~for~~ growing of groups or species of microorganisms are known currently. They include media for growth, detection and enumeration of total number of bacteria (Tryptic Soy Agar and other), fungi, molds (Sabouraud Dextrose Agar, Potato Dextrose Agar and other), selective media for group of microorganisms like Gram-negative bacteria (MacConkey Agar, Levine EMB Agar and other), *Lactobacillus* (Lactobacilli MRS Agar) or *Salmonella* (SS Agar), certain microbes like *E. coli* O:157 (Sorbitol MacConkey Agar), *Vibrio cholerae* (TCBS Agar), *Campylobacter* (Triple Sugar Iron Agar) and many others.

[004] It usually takes from several hours to several days, or even weeks to form well visible colonies or a visible suspension of cells. Growing periods on nutrient media is the most time consuming process in modern microbiological diagnostics. This significantly decreasing the time of microbiological analysis and saving the high level of reliability (i.e., detection by growth – forming of colonies) is very important for modern medical diagnostics for early diagnostics of humans or animals infections, epidemiology, and detection of antibiotic resistant microbes and so on.

[005] Rapid analysis is also needed in food, pharmaceutical, and biotechnological industries for control and prevention of contamination in food, drugs, and medical devices and for environmental monitoring.

This invention introduces a new method of rapid detection of cellular microorganisms during their preliminary short growth on or in nutrient media.

[006] The term “colony” or “micro colony” in microbiology means a group of cells appearing from one single cell and consisting only from descendants of that cell. A colony or micro colony can have different shapes: semi-sphere if grown on the surface of solid agar, oval, cone or “star” if grown in solid or semi-solid agar, a dense or diffuse cloud if grown on semi-liquid or liquid media, or flat fibers growing from one center (fungi, actinomycetes, or some bacterial micro colonies).

[007] This invention is based on changing the natural shape of micro colonies to a long cylindrical shape by growing the colony in a long and extremely small micro-channel that enhances the optical characteristics of the micro-colony and strongly improves the micro-colony’s visibility (detectability). An array of micro-channels on a glass plate is part of the Sample – Detection Unit (SDU) – a device for trapping microorganisms, allowing growth, and performing analysis. The improvement of the visibility (detectability) of the micro-colonies ~~could~~ shorten the time between inoculation and detection of the colony.

Description of the Related Art

[008] There are several different methods, instruments, and devices employed to enhance colony visibility. Thus the addition of special, non-toxic ~~for cells~~ substances (some artificial chromogenic or fluorogenic substrates) to solid nutrient media ~~could~~ change the color of the micro-colonies or makes them fluorescent and improve colonies visibility ~~on~~ in early growth stages.

[009] Some microorganisms like *E. coli* O:157, *Staphylococcus aureus*, and *Salmonella* grows on “Chromagars,” a solid or semi-solid nutrient media that specifically changes the

color of investigated microorganisms because of artificial substrates for unique enzymes added to media. These substrates are non toxic to the cells and allow normal growth. Toxic artificial substrates (example: Tetrazolium salts) such as Tetrazolium salts, Fluorescein diacetate and ~~or~~ other substances ~~could~~ can be used ~~on late~~ in later growth stage ~~of colony or suspension of cell growth~~ to colorize cells and make them more clearly visible.

[010] Detection and enumeration of the colonies are done visually or with magnifying devices. Visual detection and enumeration using magnifying glass requires relatively big colonies; from hundreds of microns to millimeters in diameter. Microscopy helps to find micro-colonies ~~with~~ of tens of microns in diameter. These colonies contain at least several hundreds of cells and need at least 5-10 hours of incubation to form colony of this size.

[011] Detection of microorganisms could also be achieved by dividing a sample into many discrete zones, adding liquid nutrient media, incubating for several hours to several days, adding indicator substance or counting turbid zones, and calculating concentration (US Patent No. 5,716,798). This method gives a reduction of growth of only 20-40% because it uses relatively large volumes of discrete zones, consisting of large wells on the side of a special flask for growth. A 90% of zone volume related to nutrient media and therefore analyzing sample could be only several milliliters. Therefore, this method is used only for the detection of microbes in human blood because blood samples are very small and their contamination is in a range from single cells to several hundred cells per milliliter.

[012] This current invention utilizes thin, long micro-channels that are open on both ends. A large volume of sample is filtrated through a micro-channel plate. The extremely small

volume of the micro-channels reduces the required growth time to 1-5 hours, an 80-95% time savings. Growth of microorganisms within these long, thin micro-channels changes the optical characteristics of micro-colonies much faster than growth of micro-colonies in a large flat volume.

[013] Thus, modern microbiology utilizes three approaches to shorten the required time ~~of needed growth and for microorganism growth and to~~ improve the visibility of colonies; first - employ optimal growth nutrient media, second – ~~addition of~~ chemical matter in the nutrient media or on a colony, or into the suspension of cells to change ~~colonies the~~ optical characteristics ~~of colonies~~, and third - employ optical instruments or devices.

[014] There are no methods utilizing the shape of the colony during its growth in order to enhance its optical density (light absorbance). Changing ~~of a~~ colony's shape from a regular semi-sphere with a large volume and a large amount of cells to a thin cylinder shape with a small volume and a small amount of cells ~~to thin cylinder shape with small volume and small amount of cells could~~ will strongly reduce the time between inoculation and colony counting. Smaller amounts of cells need a shorter time for their production. The usage of chemicals producing color or fluorescence and optical instruments and devices together with detection of cylindrical colonies ~~could~~ would improve visibility and reduce the time required for ~~of~~ analysis.

[015] ~~Reduction of the time~~ Reducing the time between the inoculation and detection of microorganisms is very important for early decisions in quality and process control in food and biotechnological industry, medical microbiology and epidemiology, air, water and surfaces control of indoor and outdoor environment, biodefense, and scientific research.

SUMMARY OF THE INVENTION

[016] The invention is based on growing of micro-colonies of microorganisms in thin and long micro-channels that are open on both ends, instead of regular growth on flat surface of solid nutrient media, or flat surface of filter placed on nutrient media, or growth in a relatively large volume of liquid nutrient media.

[017] The shape of a regular micro-colony is usually semi-sphere. The thickness (height) of micro-colonies is crucial to ~~make it visible~~ visibility using microscopy ~~because as a~~ thick (high) colony has ~~a larger~~ greater light absorbance, which is the most important optical characteristic of visibility. A long and thin micro-colony ~~have~~ has the same light absorbance as a regular semi-spherical ~~sphere~~ colony of the same height – h (see Fig. 1: 1 – regular micro-colony; 2 – micro-colony in micro-channel; 3 – filter; 4 – nutrient media). The volume and amount of cells in a long, thin micro-colony is much less, and therefore the time of incubation to create a well visible colony ~~needs to be~~ is much shorter than when growing on a flat surface.

[018] The growth of a cylindrical micro-colony ~~could be done~~ can be made with a help of a grid that has large amount of very small and long channels. The diameter of ~~this~~ each of these channels needs to be very small, only 4-20 times larger than the size of the investigated cells. A good example of a grid for this purpose ~~could be~~ is the MCGP – Micro-Channel Glass Plate. The MCGP contains thousands of extremely small, precisely ~~etched~~ manufactured long channels. A regular MCGP has a diameter of each channel 10 microns, length 500 microns, and the amount of channels *is* 700,000 channels per cm². ~~Other grids or MCGP with a similar shape and volume could also be useful.~~ In general,

the MCGP is attached to a filter (Fig 2) and a sample is filtrated through both the MCGP and filter. Some micro-channels obtain live cells.

[019] After the filtration is completed, the MCGP and filter is placed on a nutrient agar or agar block of solid nutrient media attached to this filter (Fig.5). Nutrient substances from the media penetrate the filter, and fill all micro-channels. Growth of micro colonies starts after this penetration (Fig.2).

[020] Calculations below show the obvious advantage in ~~shortening~~ reducing of the time of growth in micro-channels ~~in comparison~~ compared with flat surfaces.

[021] The regular shape of colonies growing on flat surfaces of solid nutrient media is, usually, near to semi-sphere. The volume of semi-sphere is $V_{ss} = \frac{\pi}{6} \cdot h^2 \cdot (R - h/3)$, where V_{ss} - volume of semi-sphere, R - radius of sphere and h - part of radius - height of semi-sphere.

[022] The volume of cylinder (cylindrical colony) is $V_{cc} = \pi \cdot R^2 \cdot h$, where R - radius of cylinder, h - height of cylinder.

Micro-colony with height (h) $10\mu\text{m}$ and $R=20\mu\text{m}$ has volume:

$$V_{ss} = 3.14 \cdot 10^2 \cdot (20 - 10/3) = 5234\mu\text{m}^3$$

Cylindrical colony with the same height ($h=10\mu\text{m}$) and $R=2.5\mu\text{m}$ has volume:

$$V_{cc} = 3.14 \cdot 2.5^2 \cdot 10 = 196\mu\text{m}^3$$

Thus, the volume of a cylindrical colony is smaller than the volume of semi-spherical micro-colony with the same height ~~in~~ by 27 times, ~~and~~ but both have the same light absorbance.

[023] The volume of one cell of *Escherichia coli* (*E. coli*) is near to $1\mu\text{m}^3$. The speed of multiplying of *E. coli* is around 20 min at optimal temperature, on optimal media. One cell of *E. coli* can produce 8 cells in one hour, 64 in two hours, 512 in 3 hours, 4096 in 4 hours and 32768 in 5 hours. Thus, one visible micro-colony on a flat surface, containing

5234 cells, can be formed in 4.2 hours. The cylindrical colony with the same height and light absorbance (196 cells) can be formed in 2.5 hours. Therefore, the growth of micro-colonies with a cylindrical shape has a significant advantage as visualization of colonies can be done at much earlier stages.

[024] The visualization of microorganisms in the micro-channels is much faster than in regular tubes or wells of an immunological plate, or other known laboratory devices for microorganism growth, because of the very small volume of micro-channels and *it* the long cylindrical shape. Thus, one cell in a cylindrical micro-channel, with a length 500 μm and diameter 10 μm ($V=40,000\mu\text{m}^3$), corresponds to a concentration of 25 millions cells per ml ($V=10^{12}\mu\text{m}^3$). Forty ~~40~~ cells in a micro-channel correspond to the concentration 10^9 cells per ml, which is a well-visible concentration. One cell of *E. coli* can reach this concentration (40 cells per micro-channel = 10^9 cells per ml) in 1.7 hours.

[025] Experiments show that 10 layers of colorless small cells (for example *E. coli*) are enough to find visual differences between micro-channels containing cells and empty micro-channels using a regular light microscope, ~~with even~~ with a small magnification of X100. A smaller diameter of micro-channel ~~needs~~ requires a smaller amount of cells to create 10 layers of cells in the channel.

[026] Table 1 represents the number ~~amount~~ of layers of *E. coli* that ~~could be~~ can be produced in micro-channels of different diameters ~~in different time~~ between one and five hours.

TABLE 1

Correlation between time of forming layers of cells in micro-channels and diameter of micro-channel (*E. coli*, growth at 37°C on TSA; the time of multiplication = 20 min)

Hours of Incubation Diameter of Micro channel	1 hour	2 hours	3 hours	4 hours	5 hours
2 μ k	3 layers	21	171	1365	10920
3 μ k	1	9	73	585	4680
4 μ k	0.6	5	39	315	2520
5 μ k	0.4	3	26	205	1640
7 μ k	0.2	2	13	108	860
10 μ k	0.1	1	6	50	410

[027] Table 1 shows that 10 layers of cells will be reached in a micro-channel with a diameter of 2 μ m in 1.5 hours; in a 3 μ m micro-channel in 2 hours; in a 4 μ m micro-channel in 2.3 hours; in a 5 μ m micro-channel in 2.7 hours; in a 7 μ m micro-channel in 2.9 hours and in a 10 μ m micro-channel in 3.5 hours. Thus, the detection and enumeration of long cylindrical micro-colonies, according this invention, can be done 10 – 20 times faster than regular growth, detection and enumeration of *CFU* colonies.

[028] A micro- colony can start formation regardless of initial trapping position within a micro-channel. Fig. 3 shows four typical positions of a cell in a channel. 1 – Cell was trapped on the surface of the filter and colony formed from the bottom of micro-channel. This formation appears when nutrient substances just moistens filter. 2 – Cell was trapped on the wall of micro-channel by the force of adhesion or by antibody attached to the wall preliminary to filtration.

[029] The formation of a micro-colony starts only if the micro-channel is completely filled by nutrient substances from wet agar media, or a thick paper filter filled by a nutrient broth. 3 – Cell was trapped on the filter, but was later raised up by liquid media and started forming micro-colony. In all of these cases, one cell formed a micro-colony of cells that were descendants of the first cell in the solid, semi-solid or liquid culture.

[030] The micro-channels containing colonies ~~look like~~ appear as dark dots (FIG. 6/1) when a regular light microscope is used. The addition of artificial chromo- or fluorogenic substrates to micro-colonies can reduce the time between inoculation and detection ~~color~~ ~~or fluorescence could~~ as they make micro-colonies much more visible ~~on~~ at an earlier stage.

[031] Fig. 6 shows the differences between natural non-colored micro-colonies (1), micro-colonies colored by chromogenic substrates or absorbent dyes (Fig. 6/2), and micro-colonies colored by fluorogenic substrate or fluorescent dye (Fig. 6/3). The coloration of micro-colonies inside micro-channels is done by attaching agar or filter paper treated with required substances to the opposite side of the filter attached to the micro-channel plate (Fig. 6).

[032] A light microscope sends light through the MCGP (colorless), filter (colorless), and agar (light transparent) (Fig. 6/1 and 6/2), revealing long cylindrical shaped micro-colonies because of the natural light absorption of cells, or due to cells colored by chromogenic substrates or absorbent dyes. A fluorescent microscope (Fig. 6/3) sends a shorter wave light (ultra violet, blue or other depending on dye) and accepts long waves of fluorescence (blue, green or red). Therefore, micro-channels with micro-colonies will appear as bright dots on a dark background.

[033] The structure of the SDU for fluorescent version is: MCGP (black non fluorescent), filter (black non-fluorescent), and agar (filled by fluorescent indicator). Fluorescence is considered a much more sensitive type of analysis. Thus, the micro-colonies can be much smaller/shorter than those analyzed with the use of light absorbance.

[034] This invention differs from other methods of detection of ~~CFU~~ colonies by using ~~of a~~ glass plate containing hundreds of thousands of extremely small and long micro-channels that are open from both ends (micro-channel plate). The combination of a micro-channel plate and a filter allows ~~trapping of cells~~ to be trapped on the filter surface of the filter, ~~and growing colonies inside channel~~ thus allowing colonies to grow inside the micro-channels. ~~Those~~ Colonies grown inside the micro-channels will obtain ~~high a~~ tall cylindrical shape.

[035] Tall ~~High~~ cylindrical shape of colony has long optical way (~~high light absorbance~~) but smaller volume and amount of cells which drastically reduces the time of analysis. This method ~~could be~~ is realized with a simple device consisting of a micro-channel plate ~~with channels~~, a filter to trap cells by filtration from air or liquid, and a frame ~~consists~~ consisting of ~~from~~ several parts (Fig. 4).

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[036] Fig. 1 demonstrates the geometrical differences between a regular semi-spherical micro-colony (left) and a typical micro-colony growing in a micro-channel of a MCGP. The height of both objects is equal (h) but the volume, and consequently the

number of cells and the growth time required to reach equal height, is significantly different.

Fig.2 shows three main components of the sampling-detection device: MCGP, filter and agar block (left), and several enlarged micro-channels with a growing micro-colony (right).

[037] Fig.3 demonstrates three different positions of an initially trapped cell producing a micro-colony. From left to right: growth starts from the bottom of the micro-channel on the surface of the filter (this happens when filter is moistened by media or the micro-channel is filled by media but the initial cell is attached to the filter by adhesion); growth starts at the micro-channel wall where a cell attaches by forces of adhesion or immunological reaction (micro-channel must be filled by media); cell produces other cells in liquid media, then broth fills the micro-channel separating cells; the initial cell is raised by broth to upper part of micro-channel and starts growth near the border of the liquid media and air.

[038] Fig.4 demonstrates the inner structure and general view of the sampling-detection unit (SDU), the device is used to trap cells by filtration from liquid or air, grow micro-colonies, and treat colonies with chromo- or fluorogenic substrates if needed.

[039] Fig.5 shows the process of filtration using a manifold and attaching nutrient media to the surface of the filter inside the SDU for micro-colony growth. Coloration of micro-colonies is done by agar treated with artificial substrates or dyes can be attached in the same manner.

[040] Fig. 6 shows how micro-colonies in the micro-channels appear under a regular light or fluorescent microscope. It also shows the direction of light through the micro-channels when using fluorescence microscopy.

DETAILED DESCRIPTION OF THE INVENTION

[041] This invention is based on a method and device for trapping cells from liquid or air, ~~grow relatively short time~~ allowing for cell growth in a relatively short period of time on solid or in liquid nutrient media ~~and find dark (not colored), colored or fluorescent channels that looks like large round dots under regular or fluorescent microscope,~~ treating micro-colonies with chromo- or fluorogenic substrates if desired, and analyzing micro-colonies by microscopy. Micro-channels that contain micro-colonies appear as dark, colored, or fluorescent dots under a regular light or fluorescent microscope depending on the method used.

V [042] The time of analysis ~~could be~~ can be reduced, and sensitivity can be enhanced, by ~~the usage using~~ micro-channels of smaller diameter and substances produced color or fluorescence. Physical factors like such as heating in order to coagulate proteins and increase light absorbance or light scattering, or addition of the substances produced gas bubbles within micro-channels like such as Oxygen (O₂) ~~produced from Hydrogen Peroxide (H₂O₂) by Catalase~~ could be can be employed also.

[043] A simple device for trapping cells (Sampling-detection Unit, SDU) in the micro-channels by filtration is shown in *Fig. 2 Fig.4 and Fig.5*. It consists (*Fig.4*) from a lid (1) with transparent glass or plastic, with one, two or more very small holes for respiration, a micro-channel plate (2), a filter to ~~restrain~~ trap cells (3), a holder for the filter and micro-channel plate, and a porous support (4) for the filter and micro-channel plate adjusted to the holder (6).

[044] SDU is adjusted to the regular manifold as shown in Fig. 5. The same figure shows different adjustments to the SDU mounted on the manifold for filtration. Thus a funnel for filtration of liquid samples can be adjusted to the filtrating device (left on manifold). A syringe (in the center of the manifold, Fig.5) is used for passing antibody and enzyme conjugates, if identification of micro-colonies is done by enzyme - immunological analysis, or just for passing small samples. The sampling device on the right does not have any additional devices as it is intended for air filtration – trapping bioaerosols (cells and spores) in micro-channels. After filtration is complete, the sampling device is removed from the manifold and placed on a Petri plate with nutrient media(bottom left, Fig. 5) or nutrient media agar cylinder adjusted to lower side of filter (bottom right, Fig. 5) to initiate micro-colony formation.

[045] The procedure for sampling, ~~growth~~ growing and enumeration of the micro-colonies is as following:

- A liquid or air sample containing microorganisms is filtrated through the device. The ~~cover~~ lid (Fig. 4 - 1) is taken off before filtration, and a special funnel for liquids (funnel is not ~~shown~~ presented on the picture) ~~could be~~ is adjusted. During this process, cells, if any, are caught in some of the channels of the micro-channel plate (Fig.4 - 2) on the surface of the filter (Fig.4 -3).

[046] - The support (Fig.4 - 4) adjusted to the holder (Fig.4 - 6) is removed.

- The lid (Fig.4 - 1) is placed on the device to prevent further contamination.
- The holder (Fig.4 - 5) with the micro-channel plate (Fig.4 -2) and the filter (Fig.4 -3) is placed on the surface of an eligible solid nutrient media ~~(not shown - on the picture)~~ (Fig.5, bottom – left) or in the container with a liquid nutrient media. The nutrient media ~~was~~ is absorbed by the filter and supports the growth

of a cylindrical-shaped micro-colony or penetrates through the filter in a-
channels, and supports the growth of suspended microorganisms.

[047] - The device with nutrient media is incubated~~needful time at appropriate~~
~~temperature~~ at an appropriate temperature for the required time for cell growth.

In order to ~~cut~~ reduce the time of analysis by increasing ~~of~~ light absorbance or ~~add~~
adding fluorescence, the device ~~could be~~ can be placed in the a container with an
eligible solution of artificial substrate. Otherwise the substrate can be added to
solid nutrient media in advance.

[048] - The device is placed under a light or fluorescent microscope, and the amount of
dark, colored or fluorescent channels is detected and enumerated. This amount ~~that~~
corresponds to the number of cells trapped on the surface of the filter. The
difference between non-colored micro-colonies, micro-colonies colored by
chromogenic substrates or colored dyes, and micro-colonies colored by fluorogenic
substrates or fluorescent dyes is shown in Fig. 6.

[049] This invention is capable of detecting a range of microorganisms from a single cell
to several hundreds of thousands or even millions, depending on the number of micro-
channels in the MCGP. For example, a 25 millimeter square MCGP with micro-channels
10 microns (μm) in diameter (square of plate around 5 cm^2 and 700,000 micro-channels
per one cm^2) contains 3.5 millions of micro-channels.

[050] Thus, in order to have a reliable enumeration of microorganisms when analyzing
the sample, the number of live microbes in a sample should not exceed 350,000 for this
plate so that one cell goes to one micro-channel with high level of reliability. If a sample
is expected to contain a higher concentration of microbes, it can be diluted. In
comparison, a regular Petri plate limits colony growth from one single cell to only 300

cells/colony as recommended by US Food and Drugs Administration, otherwise colonies will begin to overlap each other and decrease the reliability of enumeration.

[051] This method and device ~~could be~~ can be used with a broad range of different solid and liquid, natural or artificial media and a large number of different samples. ~~A micro-channel plate without a filter can be used to find contamination on surfaces by spraying liquid nutrient media on the surface and placing the micro-channel plate above, incubating the required amount of time and reading the results under a microscope. This invention can be realized in many different optical or opto-electronic instruments and devices.~~

Example 1

Detecting and enumerating live bacteria in liquid samples

[052] Detecting and enumerating bacteria in samples is one of the most widely used analyses in medical, environmental and industrial microbiology. Liquids tested include drinking water, beverages, industrial liquids used for manufacturing, natural water, urine, spinal and other body fluids, wash out from surfaces and so on. One of the mostly used nutrient solid media for detection of total viable organisms (mainly bacteria) is Tryptic Soy Agar (TSA). Regular growth of colonies on a Petri plate filled with TSA requires 24 to 72 hours at 35°C. Using the proposed invented method; incubation requires only 4 hours.

[053] The procedure for the invented technology is as follows:

- The sample (100 milliliters) is filtrated through the SDU, trapping cells in micro-channels containing a colorless MCGP and colorless filter (Polycarbonate, 0.2 microns

pores, Osmonics Inc, USA) (Fig. 4, Fig.5). Live cells, if any in the sample, are trapped in some of the micro-channels.

[054] - The SDU is removed from the manifold and a nutrient media (TSA) agar block is attached to the surface of the filter. Nutrient substances from the TSA saturate the filter and penetrate into micro-channels. This process takes around 10-30 seconds.

- The SDU with attached agar block is placed in an incubator for 4 hours at 35°C. Long cylindrical micro-colonies from individual trapped live cells form.

[055] - After incubation, the nutrient media agar block is removed. Another agar block containing Thiazolyl blue tetrazolium salt is adjusted to the filter (a thick filter paper filled by an indicator substance can be used instead). Yellowish molecules of Thiazolyl blue penetrates into the micro-channels. Any micro-channels containing micro-colonies become dark violet in color. The reaction of tetrazolium salt with cells is well-known and based on dehydrogenases of living cells accepting a Hydrogen atom (H⁺) from the tetrazolium ring for further use in respiration.

[056] This reduction then results in a colored substance (Formazan) that collected inside live bacterial cells, mainly in mesosomes. All known bacterial and fungal cells react with tetrazolium salt to reveal this color reaction. Intensely colored long cylindrical micro-colonies are more visible than the same non-colored micro-colonies or colored flat micro-colonies (grown without micro-channels).

[057] An agar block (2% in distilled water, 1 cm³ volume) can be prepared with Thiazolyl blue by adding 3-4 drops of a 3 milligrams per milliliter Thiazolyl blue in phosphate buffer (pH=7.2). Intensely colored cylindrical micro-colonies are visible as colored circles and are easily enumerated in a regular light microscope with a

microscopic multiplication from X40 or larger. The concentration (cells per milliliter) of live cells in a sample is calculated by regular known methods.

Thus detection and enumeration of microorganisms in a sample can be completed 6 – 12 times faster than when depending upon cell growth on a regular Petri plate.

[058] Other indicators of live cells can also be used:

- Colorless Fluoresceine diacetate or butirate cleaves by Esterases with the release of highly fluorescent Fluoresceine (green fluorescence = 515 nm). Fluoresceine collects (crystallizes) inside cells and interrupts biochemical pathways, which cause cell death. Thus, Fluoresceine diacetate and other Fluoresceine derivatives can be used after micro-colonies are formed.

[059] - Colorless 4-Methylumbelliferyl acetate, -butyrate, -propionate, or -phosphate cleaves by Esterases, Lipases or Phosphatases with the release of 4-Methylumbelliferone, a highly fluorescent substance (blue fluorescence = 450 nm). 4-Methylumbelliferone is secreted from cells and concentrates in extracellular spaces, filling the remaining volume of the micro-channel. Therefore, these fluorogenic substrates can be used during micro-colony growth. Simultaneously incubating a sample and allowing cell growth while treating cells with 4-Methylumbelliferone, and thus allowing an extracellular buildup of fluorescent signal, can significantly reduce the time required for analysis as very small micro-colonies (10-20 cells) can be detected.

[060] - Other than Thiazolyl blue, Tetrazolium salts such as Tetrazolium iodo (INT), Nitrotetrazolium blue (NBT), and BT-tetrazolium that reveal differently colored Formazans can also be employed. Nevertheless, these salts are not as universal as Thiazolyl blue and, therefore, can be used for certain samples.

[061] - Chromogenic substrates such as 5-Bromo-4-chloro-3-indoxyl butyrate, -palmitate, -phosphate (blue precipitates inside cell) or 6-Chloro-3-indoxyl butyrate, -palmitate (red color precipitates) for Esterases, Lipases, or Phosphatases, as well as other chromogenic substrates can be used for coloration of formed micro-colonies and as additives to nutrient media. Chromogenic substrates that are dissolved in nutrient media before application for cell growth are referred to as “Chromagars.” However, Chromagars are created for only a few microorganisms: CHROMagar™ Candida, CROMagar™ O157, CHROMagar™ Salmonella, CHROMagar™ Staph aureus and CHROMagar™ Orientation for urinary tract pathogenic microorganisms.

[062] - Dyes, such as Dansylchloride (DNS-chloride) or Fluorescamine, are capable of increasing fluorescence hundreds- or even thousands-fold after attaching to biomolecules such as NH- groups of proteins. These compounds are also useful for marking micro-colonies for enumeration.

[063] - Some substances are known to change the color of colonies to a dark or even black color, making micro-colonies more visible on a Petri plate or under a microscope in micro-channels. For example, iron sulfide in SPS Agar is known to color *Clostridia*, and XLT4 Agar Base colors *Salmonella*. Likewise, potassium telluride in VJ Agar colors *Staphylococcus aureus* colonies.

[064] Growth of more than 4 hours is often enough to produce long micro-colonies, and detect and enumerate a large number of non-colored micro-colonies by their enhanced light absorbance or light scattering. Also, light absorbance and light scattering of micro-colonies in micro-channels can be increased by high temperature (proteins coagulation) or addition of reagents that produce bubbles in the presence of live cells (i.e., Catalase –

Hydrogen Peroxide) and are known to have good refractivity and therefore light scattering.

[065] Many different kinds of samples, a variety of microorganisms, hundreds of nutrient media, and a multitude of indicator substances opens a huge opportunity for the implementation of this invented technology in the different areas of microbiology.

Example 2.

Identification of micro-colony of E.coli O:157 by enzyme immunoassay in micro-channels

[066] Micro-colonies can be identified in the micro-channels using enzyme immunological analysis (EIA). Cells are trapped in the micro-channels by filtering a sample as described in Example 1. The use of EIA for the identification of micro-colonies is based on the immunological reaction between antigens of the cells (micro-colony) and enzyme-antibody conjugates. The conjugate is passed through the micro-channels in order to perform antibody – antigen reactions. The syringe shown in Fig.5 is suitable for this because it allows a small volume of conjugate to be slowly pressed through the micro-channels. The micro-colonies in this case must be very small, 8-32 cells, as larger micro-colonies can clog the micro-channel.

[067] After the conjugate attaches to *E. coli* O:157 antigens, a block of pure agar filled by Tetramethylbenzidine (a substrate for Horseradish Peroxidase – an enzyme of the conjugate) is attached to the filter. Tetramethylbenzidine is cleaved by the Horseradish Peroxidase with creation of a blue-colored dye that collected in micro-channel with *E.coli* O:157. The number of *E.coli* O:15 present in the sample corresponds to the number of

blue micro-channels. This example is based on a well known color reaction, but fluorescent reactions are also available. Thus a conjugate consisting from antibody and β -D-Galactosidase gives a fluorescent 4-Methylumbelliferone in reaction with 4-Methylumbelliferyl- β -D-Galactose. Conjugates consisting from an antibody and Phosphatase produce 4-Methylumbelliferone in reaction with 4-Methylumbelliferyl phosphate, disodium salt.

Example 3

Sampling, detection and enumeration of bioaerosols.

[068] MCA technology can also be used for the rapid analysis of bioaerosols. Air is filtrated through the SDU, which is adjusted to the manifold (Fig. 6, right holder). The manifold is connected to an air pump (AirCheck HV30, QuickTake 30 or another, SKC Inc., USA). A rotameter for measuring the air volume is installed between the manifold and the pump. A required volume of air is passed through the SDU, and microorganisms present in the air sample are trapped in the micro-channels. Bacilli and Fungi spores are considered the main microorganisms in bioaerosols.

[069] Thus, two nutrient media need to be used, TSA for Bacilli and SDA for Fungi spores. This also requires using two SDU. Dormant spores of Bacilli usually germinate within 0.5-1.5 hours after contact with a nutrient media. This time needs to be added to the regular time of Bacilli incubation trapped in micro-channels (4 hours) in order to form a cylindrical micro-colony from the spore. Germination of Fungi spore requires about 2-8 hours, followed by incubation in the SDU of about 12 hours. After micro-colonies from spores appear in the micro-channels, procedures described in Example 1 (detection of the

total number of viable microorganisms) or Example 2 (identification of micro-colonies) or other procedures developed for micro-channel analysis take place.

[070] Another version of sampling air microorganisms is by first sampling in liquid (sodium chloride solution, buffer, liquid nutrient media) with help of well-known liquid samplers (e.g., AGI-30, SKC BioSampler) and then filtrated through the SDU. Currently used methods for bioaerosol detection are based on “landing” particles on the surface of agar media (Impactor BioStage, SKC Inc.) or inoculating liquid samples with microorganisms sampled beforehand. Both methods need a long growth period for the microorganisms in order to form well visible colonies: 24-72 hours for bacteria and 72-120 hours for fungi.

Appl. No. 10,628,110

Admt. dates Jul. 28, 2003

Reply to Office action of Mar. 7, 2005

ABSTRACT

(Revised)

~~The time of the detection and enumeration of microorganisms after their growth on solid or liquid nutrient media depends on the visibility of colony or suspension by naked eye or optical instruments. Visibility depends mainly on light absorbance by layer of cells in colony or suspension. The growth of microorganisms in micro channels needs much less amount of cells to reach the same light absorbance as done by regular growth. Smaller amount of cells needs shorter time for their reproduction. Therefore detection and enumeration of cells could be done in several times faster than by previously known growth methods. Also the time of detection and enumeration could be shortened by additional usage of chemical substances or physical factors that increase light absorbance or instill fluorescence. To reach needful light absorbance the volume of one micro channel must be extremely small—only in several thousands times larger than the volume of one cell and longevity of channel must be in several times longer than diameter of a channel.~~

This invention describes a method of rapid detection of micro-colonies of microorganisms by changing their shape from a regular semi-sphere to a long and thin cylinder. Cells are trapped by filtration in long (diameter/length= 1/10 – 1/100).

cylindrical, parallel, micro-channels (1-500 picoliters each) that are open from both sides, and attached to a filter from one side. A micro-channel plate houses a multiplicity of micro-channels (diameter of each channel = 1 – 20 μm , and length 100 – 1000 μm). The micro-channel plate with cells trapped on the surface of the filter is attached to a nutrient media agar block. Cells produce micro-colonies of a long and thin shape according the shape of the micro-channel. The growth of microorganisms in the micro-channels permits a change in the number of cells to accomplish light absorbance. Fewer cells need a shorter time to reproduce. Thus detection and counting of cells can be accomplished in a rapid fashion. The light absorbance can be enhanced by additional coloration of micro-colonies by cell dyes or artificial chromogenic or fluorogenic substrates.